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Teaser Novel drugs and novel excipients in pH-dependent ileocolonic drug delivery systems have to be tested in animals. Which animal species are suitable and what in vivo methods are used to verify ileocolonic drug delivery?



pH-dependent ileocolonic drug delivery, part II: preclinical evaluation of novel drugs and novel excipients

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Introduction

In part I of this review series we described *in vitro* and clinical methods to investigate and verify colonic drug delivery of novel pH-dependent systems [1]. These systems utilize the sharp but short pH peak of 7.4 (range 7.2–7.7) in the ileum for ileocolonic drug targeting. When evaluating the ileocolonic targeting ability of a system or the therapeutic efficacy of an ileocolonic delivered drug, it is preferable to test it directly in humans [1]. However, a novel drug and/or a novel excipient cannot be tested in humans if no safety data from animal studies are available for the drug or excipient used for the ileocolonic drug delivery systems [2]. Guidelines concerning safety testing have been provided by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [3]. According to these guidelines, safety pharmacology studies have to be conducted with the final formulation in laboratory animals if the formulation substantially alters the pharmacokinetics and/or pharmacodynamics of the drug compared with previously tested formulations [4]. Given that ileocolonic drug delivery systems target the drug to the lower parts of the gastrointestinal (GI) tract, both the pharmacokinetics and pharmacodynamics of the drug will be altered, thus necessitating safety testing with the final drug delivery system in laboratory animals. To achieve pH-dependent ileocolonic drug delivery in animals, the animal species chosen should have a sharp distinct pH peak in its terminal ileum. Ideally, the intestinal pH values of the animal should be comparable with those found in humans, including the pH peak above pH 7.2 in the terminal ileum. This would allow the use of established pH-dependent ileocolonic targeted drug delivery systems to test novel drugs. To test novel pH-dependent excipients, the pH of the GI tract of the chosen animal species has to be similar to that of humans to obtain ileocolonic targeting in humans. Various species have been used for preclinical testing of new drugs or novel excipients, including rats, mice, dogs, and rabbits. However, little emphasis has been put on the translation from animal species to humans regarding the pH values of the GI tract.

In this review, we provide an overview of the pH values of various parts of the GI tract of frequently used laboratory animal species and of humans. We aim to determine which animal species, if any, could best be used to test novel drugs or novel excipients in pH-dependent

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ileocolonic drug delivery systems. Subsequently, *in vivo* methods used in preclinical evaluations to investigate and verify ileocolonic drug delivery are discussed.

Luminal pH in the gastrointestinal tract of animals compared with humans

The pH values of the GI tract largely determine in which part of the GI tract a drug is released from a pH-sensitive drug delivery system. For ileocolonic drug delivery, it is important to select an animal species that has a sharp and distinct pH peak in the terminal ileum, as is found in the human GI tract [1]. In addition to pH, other factors can have a role in the performance of pH-dependent ileocolonic drug delivery systems, including intestinal length, buffer capacity, fluid volume, motility, and transit time. These factors have been extensively described by Hatton *et al.* and Sjögren *et al.* [5,6], and are not further discussed in this review.

Methods to determine the pH in the gastrointestinal tract in laboratory animals

Similar to human studies, pH values in the GI tract of animals have been determined with aspiration via the oral route, tethered pH electrodes, and with pH-sensitive radio telemetry capsules. In humans, aspiration via colonoscopy has also been used to measure the pH of the lower GI tract, but to our knowledge this method has never been used in animals [1]. Aspiration via the oral route is generally limited to the upper GI tract and can be used to determine the mean pH of the collected stomach or duodenal fluid *ex vivo* [7,8]. If the pH of the entire GI tract during transit is to be measured, radio telemetry capsules can be used [9–11]. A drawback of these capsules is their large size (usually around 10 × 20 mm), which limits their use to larger animals, such as dogs and pigs (Table 1) [12–15]. With laboratory animals, *ex vivo* pH measurements of the intestinal contents after surgical collection of samples from different segments of the GI tract is possible [16–23]. A drawback of this method is that only the mean pH of the collected fluid is determined. In addition, the pH should be measured immediately after collection to prevent possible postsampling pH changes. This is particularly relevant for the content of the colon, because bacterial fermentation of polysaccharides results in the formation of acidic products which can lower the pH after collection [24]. Another option to measure the pH in animals is to open the GI tract by surgery and to measure the pH *in situ* with a pH electrode [25–32]. With *in situ* pH measurements and with a pH-sensitive radio telemetry capsule possible pH alterations after sampling are excluded, because the pH values are measured immediately.

pH values in the gastrointestinal tract of laboratory animals

The mean or median pH values found in various studies for different segments of the GI tract (stomach, duodenum, jejunum, ileum, cecum, and colon) of rabbits, pigs, dogs, rats, mice, guinea pigs, and monkeys are shown as dots in Fig. 1, together with the values for humans as reviewed in part I of this series [1]. From publications in which both the median and mean pH values were given, the mean values were used in this review. When multiple pH values were reported for a certain GI tract region, for instance the fundus and antrum of the stomach, the mean value was calculated and used. In Fig. 1, the minimum and maximum pH

values are indicated by bars and the mean pH values, calculated from all different studies combined, by a vertical line. The study size was not taken into account for the calculation of the mean. In this overview, no differentiation is made between the fasted and fed states, because of a lack of sufficient data on this point. To enable comparison, the previously reported values for humans in the fasted and fed states were also combined. Table 1 provides an overview of the studies used, with information about the pH measurement method and the state (fasted or fed) under which the experiments were carried out.

Rabbits and pigs have pH values in the stomach that are within the range of the human values (Fig. 1A,B, respectively) [16–18,25,33]. The mean pH values of the duodenum and jejunum in these animal species are below the minimum pH of 7.2 that is found in the human ileum, whereas the pH values in the ileum were higher [16–18,25,33].

A broader variation was found for the pH in the stomach of dogs (pH 1.1–6.8; Fig. 1C) and rats (pH 3.2–6.7; Fig. 1D) [7–11,16,18,21,23,26,28–32]. In these animals, the minimum pH of 7.2 of the human ileum is already surpassed in the duodenum and jejunum in some of the studies. Additionally, the mean pH in the ileum of both dogs and rats was below this minimum pH of 7.2 [7,16,18–21,23,26,28–30].

Mice have a higher pH in the stomach but a lower pH in the small and large intestine compared with humans (Fig. 1E) [16,22,23,28]. The minimum pH of 7.2 found in the ileum of humans was not reached in any part of the murine GI tract.

In guinea pigs, the pH values of the GI tract are higher than those of humans (Fig. 1F). The minimum pH of 7.2 was reached in the duodenum in some of the studies and was above pH 7.2 in the jejunum in all studies until the cecum, where the pH drops until 6.7 [16,25].

In monkeys, the mean pH in the stomach was higher than in humans (Fig. 1G) [16,34]. The pH increases to pH 6.0 in the ileum and then drops to 5.0 in the cecum and colon [16]. However, care must be exercised to draw definite conclusions from these data because the pH values of the small intestine and colon are based on only one study.

Selection of an appropriate animal species

For the preclinical evaluation of novel drugs or novel excipients applied in pH-dependent systems, we found that no particular animal species is commonly used (Table 2). It is remarkable that in most studies no information is given about the rationale behind the chosen animal species. In view of the working principle of pH-sensitive ileocolonic targeted drug delivery systems, a pH peak in the terminal ileum of the animal should be considered as the most important factor. Furthermore, pH values similar to the human GI tract would be ideal, because in that case an existing and well-validated pH-dependent system can be used to obtain ileocolonic drug delivery in the chosen animal species or novel pH-dependent excipients can be evaluated.

The pH profile of the GI tract of monkeys, mice, and guinea pigs differs from that in humans and no distinctive pH peak has been found in the terminal ileum. Therefore, these laboratory animal species should be considered unsuitable for testing novel drugs in pH-dependent ileocolonic drug delivery systems (Fig. 1). Dogs and rats might be suitable because the mean pH values in the various

TABLE 1

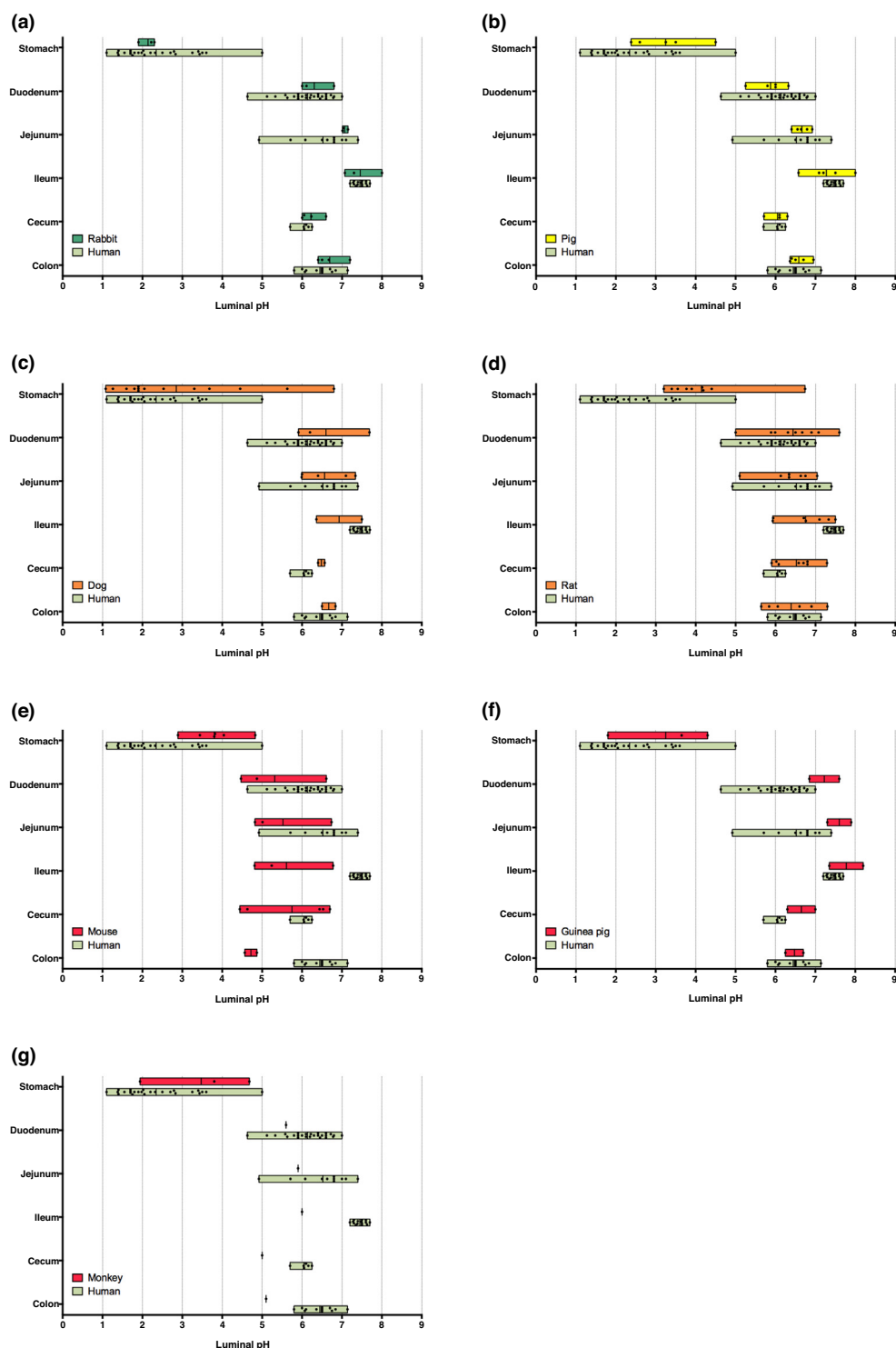
Overview of studies investigating the pH values of the GI tract in laboratory animals and in healthy human individuals

| Animal | | Segment gastrointestinal tract | | | | | | Method | | Refs |
|------------|--|--------------------------------|----------|---------|-------|-------|-------|--|--|-------|
| Species | Breed | Stomach | Duodenum | Jejunum | Ileum | Cecum | Colon | pH measurement | Fasted/fed | |
| Monkey | Cynomolgus | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fed <i>ad libitum</i> | [16] |
| | | X | | | | | | Bravo TM capsule ^b | Fasted and fed | [34] |
| Pig | From farms | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fed <i>ad libitum</i> | [16] |
| | Crossbreed of large white and Landrace | X | X | X | X | X | X | <i>In situ</i> ^a | Fed <i>ad libitum</i> | [25] |
| | | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fed <i>ad libitum</i> | [18] |
| | Large white, Landrace, and Essex | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fasted for 1 hour | [17] |
| Mouse | Balb/c | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fasted and fed | [23] |
| | House | X | X | X | X | X | | <i>In situ</i> ^a | N/A ^e | [27] |
| | | X | | | | X | | <i>Ex vivo</i> ^a | Fed <i>ad libitum</i> ; high and low fiber diet | [22] |
| | White | X | | | | | | <i>Ex vivo</i> ^a | Fed <i>ad libitum</i> | [16] |
| Rat | Albino Norwegian | X | X | X | X | X | | <i>In situ</i> ^a | Fasted and fed <i>ad libitum</i> | [28] |
| | Porton-Wistar | X | X | | | X | X | <i>In situ</i> ^a | Fasted, fed <i>ad libitum</i> and fed standardized | [29] |
| | | X | X | | | X | X | <i>In situ</i> ^a | Fed | [26] |
| | White | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fed <i>ad libitum</i> | [16] |
| | Wistar | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fasted and fed | [23] |
| | | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fed <i>ad libitum</i> | [18] |
| | | X | X | X | X | | | <i>In situ</i> ^a | Fasted | [30] |
| Rabbit | New Zealand Whites | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fed <i>ad libitum</i> | [16] |
| | | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fed <i>ad libitum</i> | [18] |
| | | X | X | X | X | X | X | <i>In situ</i> ^a | Fed <i>ad libitum</i> | [25] |
| | | | | | | X | X | N/A ^e | Fed <i>ad libitum</i> | [33] |
| Guinea pig | N/A ^e | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fed <i>ad libitum</i> | [16] |
| | Dunkin-Hartley White | X | X | X | X | X | X | <i>In situ</i> ^a | Fed <i>ad libitum</i> | [25] |
| Dog | Beagle | X | | | | | | Heidelberg capsule | Fasted | [9] |
| | | X | | | | | | Aspiration | Fasted | [8] |
| | | X | | | | | | <i>In situ</i> ^a | Fasted | [31] |
| | | X | | | | | | Bravo TM capsule | Fed big or small meal before capsule ingestion | [10] |
| | | X | | | | | | Bravo TM capsule | Fasted, fed standard meal and fed slurry meal | [11] |
| | | X | | | | | | <i>In situ</i> ^a | Fed | [32] |
| | From domestic households | X | X | X | X | X | X | <i>Ex vivo</i> ^a | Fed <i>ad libitum</i> | [16] |
| | Labrador | X | | X | | | | Aspiration | Fasted | [7] |
| | | | | X | | | | <i>Ex vivo</i> ^a | Fasted and fed | [20] |
| | Mixed | | X | | | | | <i>Ex vivo</i> ^a | Fasted | [19] |
| | N/A ^e | X | X | X | X | X | X | <i>Ex vivo</i> ^c | Fasted | [21] |
| Human | | X | X | X | X | | X | Telemetry capsule | Fasted; food when capsule left stomach | [105] |
| | | X | X | X | X | | X | Bravo TM capsule | Fasted; food 30 min or 4 h after ingestion | [106] |

TABLE 1 (Continued)

| Animal | | Segment gastrointestinal tract | | | | | | Method | | Refs |
|---------|-------|--------------------------------|----------|---------|-------|-------|-------|--|---|-----------|
| Species | Breed | Stomach | Duodenum | Jejunum | Ileum | Cecum | Colon | pH measurement | Fasted/fed | |
| | | X | X | X | | | | <i>In situ</i> ^a | Fasted | [38] |
| | | X | X | | X | X | X | Telemetry capsule | Fasted; food allowed when capsule left stomach | [40] |
| | | X | X | | X | | X | Intellipac [®] capsule | Fasted; food allowed 4 h after ingestion | [107] |
| | | X | X | | | | | Heidelberg capsule | Fasted and fed | [108] |
| | | X | X | | | | | Aspiration | Fasted | [109] |
| | | X | X | | | | | Aspiration | Fasted | [110] |
| | | | X | | X | | X | SmartPill | Fed; FDA standard breakfast | [111,112] |
| | | | X | | X | | X | SmartPill | Fasted; after 4.5 h standardized lunch | [111] |
| | | X | | X | X | X | X | Telemetry capsule | Fed; food allowed after ingestion of capsule | [36] |
| | | X | | | | X | X | IntelliCap [®] | Fasted; food 3, 6 and 10 h after ingestion | [92] |
| | | X | | | | | X | SmartPill | Fed; gastric-emptying meal | [113] |
| | | X | | | | | | Heidelberg capsule | Fasted; food 3 h after capsule left stomach | [9] |
| | | X | | | | | | <i>In situ</i> ^a | Fasted and fed standard meal | [114] |
| | | X | | | | | | Aspiration | Fasted | [115] |
| | | X | | | | | | <i>In situ</i> ^a | Fasted | [116] |
| | | X | | | | | | Aspiration | Fasted | [117] |
| | | X | | | | | | <i>In situ</i> ^a | Fasted and fed | [118] |
| | | X | | | | | | Heidelberg capsule | Fed; standardized breakfast | [119] |
| | | X | | | | | | <i>In situ</i> ^a | Fasted and fed | [120] |
| | | X | | | | | | <i>In situ</i> ^a | Fasted and fed | [121] |
| | | | X | X | X | X | X | Telemetry capsule | Fasted; food allowed when capsule left stomach | [122] |
| | | | X | X | X | | X | Telemetry capsule | Fed; normal diet | [123] |
| | | | X | X | X | | X | Telemetry capsule | Fasted; food allowed when capsule left stomach | [37] |
| | | | X | X | | | | Aspiration | Fasted | [124] |
| | | | X | | X | | | Heidelberg capsule | Fed | [125] |
| | | | X | | | | | Aspiration | Fasted, fed and fed fat-enriched meal | [126] |
| | | | X | | | | | Bravo TM capsule ^d | Fed; standardized meal twice daily | [127] |
| | | | X | | | | | Aspiration | Fasted | [128] |
| | | | X | | | | | Aspiration | Fasted | [129] |
| | | | X | | | | | Aspiration | Fasted and fed | [130] |
| | | | | X | X | | X | Telemetry capsule | Fasted; food allowed when capsule left stomach | [131] |
| | | | | X | X | | X | SmartPill | Fasted; food allowed 6 h after ingestion | [132] |
| | | | | | X | X | | SmartPill | Fasted; standardized meal when capsule left stomach | [133] |
| | | | | | X | X | | SmartPill | Fed; test meal, after 6 h normal diet | [39] |

^a Measured with a pH electrode.^b Capsule attached to stomach.^c Colorimetric and with a pH electrode.^d Capsule attached to duodenal bulb.^e Not available.



Drug Discovery Today

FIGURE 1

Luminal gastrointestinal (GI) pH values of laboratory animals (dark-green, orange, or red bars) and healthy human individuals (light-green bars). The different pH values of the GI tract of the (a) rabbit [16,18,25,33], (b) pig [16–18,25], (c) dog [7–11,16,19–21,31,32], (d) rat [16,18,23,26,28–30], (e) mouse [16,22,23,27], (f) guinea pig [16,25], and (g) monkey [16,34] are given against the values in healthy human individuals [9,36–40,92,105–133]. The GI pH ranges are indicated by bars, the vertical line in the bars indicates the mean pH. The mean or median pH values of the different segments of the GI tract found in the different studies are indicated by the black dots. Only one study was found for pH values for the duodenum, ileum, cecum, and colon in the monkey and, therefore, only this value is given as a dot and a vertical line. Table 1 in the main text details the studies that were used to obtain the minimum, maximum, and mean pH values for the different segments of the GI tract.

segments of their GI tract are generally comparable to those in humans, including a pH peak in the terminal ileum (Fig. 1). However, their mean pH value in the ileum is below the minimum value in humans. Furthermore, the pH values in the GI tract substantially varied among the different studies. Therefore, it is advised to check the inter- and intraindividual variation in pH values of the GI tract of the dogs or rats used in the experiments. Given the relatively broad range of gastric pH values in dogs, it is recommended to pretreat the animals with a 0.1 M HCl-KCl solution, via an orogastric tube, to lower the pH of the stomach [35]. Rabbits and pigs have pH values comparable to those in humans, including the pH peak in the ileum above pH 7.2 (Fig. 1). The pH values in the different segments of the GI tract showed relatively little variation between the different studies. For rabbits, the mean pH in the jejunum was slightly higher (7.1) than in humans, which might result in premature drug release. However, the rabbit is the only animal species having a distinct pH peak in the ileum, with the minimum pH above 7.0. For pigs, the minimum pH found was 6.6, which is considerably lower. In four out of five pig studies, a pH in the ileum above 7.0 was found, whereas this was the case for all three rabbit studies. Thus, when using pigs, it is advised to check the pH values of the GI tract of the individual animals to verify whether they are comparable to those of humans and constant over time.

Overall, the rabbit appears to be the most reliable species for testing novel drugs with established pH-dependent ileocolonic drug delivery systems because of the low pH variability and the distinct pH peak above 7.0 in the ileum. When the inter- and intraindividual variations in pH values of the different regions of the GI tract are checked for rabbits, ileocolonic drug targeting might be proved with even more certainty. In cases where the pH values of the GI tract for individual pigs or dogs are comparable to those in humans, are constant over time, and if the pH threshold of the system is reached, these animal species might be good alternatives to the rabbit, because larger drug delivery systems can be administered. When multiple animal species are found to have appropriate pH values in the GI tract, pilot studies could be performed to determine which species is most optimal to obtain ileocolonic drug delivery with the chosen drug delivery system. To test the therapeutic effect of a novel drug in preclinical studies (Table 2), diseased rather than healthy animals should be used. However, a diseased state could change the pH values in the GI tract in animals, even though it was found that colonic diseases did not negatively affect the pH values in humans [1,36–40]. However, despite these results, it is still recommended to check the pH values of the GI tract of diseased animals, because the effects of a colonic disease on the intestinal pH have not yet been described for animals.

It should also be possible to mimic a specific disease in the chosen animal species to enable measurement of the therapeutic effect of a novel drug. In the literature, several colonic disease models have been described for the rabbit (Table 3), which, according to our review, is the most reliable animal species to test novel drugs in a pH-dependent ileocolonic drug delivery system. Another important aspect to be taken into consideration is that the pharmacokinetics and/or pharmacodynamics of a drug might be different in an animal compared with humans. Thus, an animal species should be selected with care, to avoid false negative or false positive results.

***In vivo* methods to investigate or verify ileocolonic targeting in laboratory animals**

Before performing *in vivo* studies, the pH dependency of a chosen delivery system should be verified in a challenging *in vitro* dissolution test. The dissolution test should mimic the pH profile and preferably the buffer capacity, buffer type, and ionic strength of the human GI tract, as described in part I of this series [1]. To draw conclusions from the *in vivo* efficacy data of a novel drug or the targeting ability of a drug delivery system containing a novel pH-dependent excipient, it is important to verify ileocolonic drug delivery in the chosen animal species. Different methods have been used to verify ileocolonic drug delivery in animals. These methods include investigation of tissue samples and utilization of imaging techniques, such as X-ray imaging (radiography and fluoroscopy), γ -scintigraphy, fluorescence microscopy, and near-infrared (NIR) fluoroscopy. Furthermore, drug plasma concentrations and therapeutic effects have been used. These methods are discussed in more detail in the following sections.

Harvesting tissue samples

After sacrificing an animal, tissue samples can be collected in which the drug concentration is determined or in which the drug delivery system and/or the drug is detected with micro- or macroscopic techniques. Different sections of the GI tract can be removed from the animal, such as the stomach, small intestine, cecum, and large intestine. Four different methods have been developed to analyze harvested tissue samples (Table 4). All of these methods are applicable for drugs that are absorbed into the systemic circulation, but, under certain conditions, three of them can be used for drugs that are not absorbed from the GI tract.

In the first method (i) the intestinal content is immediately washed away. Subsequently, the drug, if present, is extracted from the tissue by an appropriate method and quantified [41–45]. With this method, drug release is indicated by the presence of drug in the extract because only released drug can be absorbed by the intestinal tissue. In the second method (ii), the washing step is omitted and the luminal fluid is included, next to the intestinal tissue, in the extraction procedure [46,47]. To draw conclusions from the drug content data, it is important to validate the extraction procedure and to determine whether the drug is completely extracted from the drug delivery system or not at all. If the drug is indeed completely extracted (iia) from the dosage form, drug release is indicated by incomplete recovery because, in that case, part of the drug has been absorbed into the systemic circulation [47]. If the drug is not extracted from the dosage form (iib), then presence of the drug in the extract is indicative of drug release [46]. In the third method (iii), only the drug content in the luminal content is measured while the tissue itself is not used [48]. For this method, it is also important to know whether the drug is completely extracted from the drug delivery system or not at all. If the drug is completely extracted (iia), incomplete recovery indicates drug release. If the drug is not extracted (iib), the presence of the drug in the extract indicates drug release. In the fourth method (iv), the drug delivery system is retrieved from the luminal content and only the drug content in the delivery system itself is determined [49]. In this method incomplete recovery indicates drug release.

In three of the different approaches, the location of release of drugs that are not absorbed into the systemic circulation can be

TABLE 2

Overview of pH targeted ileocolonic drug delivery systems tested in laboratory animals

| Animal | | | Drug delivery system | | In vivo test | | Refs |
|-------------|-----------------------------------|------------------------------|---|---------------------------------|--|--|-------|
| Species | Rationale | pH values GI tract mentioned | pH-dependent matrix (M) and/or coating (C) | Drug/marker | Read out | Colon arrival determined with | |
| Dog | N/A ^a | Yes | Eudragit S (C) | Mesalazine; tegafur | Plasma samples | Plasma concentrations compared with previous determined colon arrival time | [77] |
| | Based on practical considerations | Yes | Eudragit FS 30 D (C) | Meloxicam | Plasma samples | Plasma concentrations and colon arrival time from literature | [78] |
| | GI tract comparable to human | Yes | Eudragit S (C) | Mesalazine | Tissue sections harvested; plasma samples | Plasma and tissue concentrations | [42] |
| Dog and rat | Pharmacokinetics | No | Eudragit FS 30 D | Lovastatin | Plasma samples | N/A | [134] |
| | N/A | No | Eudragit S (M) | Insulin; salicylic acid | Plasma samples and tissue sections harvested | Plasma concentrations and visual examination of harvested tissue sections | [53] |
| | GI tract comparable to human | Yes | Eudragit P-4135 F (C); Eudragit L (C); Eudragit S (C) | Norfloxacin; fluorescein | Tissue sections harvested; plasma samples | Sulfasalazine method | [52] |
| Rat | N/A | No | Eudragit S (C) | Mesalazine and/or curcumin | Colitis severity | N/A | [135] |
| | N/A | No | <i>N</i> -succinyl chitosan/Zn ²⁺ (M) | Mesalazine and/or zinc | Colitis severity | Colitis severity and <i>in vitro</i> release | [99] |
| | N/A | No | Acrylic acid and butyl meth-acrylate polymers (M) | Aceclofenac | Colitis severity | N/A | [136] |
| | N/A | No | Eudragit S (M) | Celecoxib and/or curcumin | Colitis severity | N/A | [137] |
| | N/A | No | Poly(starch/acrylic acid) (M) | Rutin | Colitis severity | Colitis severity and <i>in vitro</i> release | [100] |
| | N/A | No | Eudragit P-4135 F (M) | Tacrolimus | Colitis severity, plasma samples | N/A | [138] |
| | N/A | No | Eudragit P-4135 F (M) | Tacrolimus | Colitis severity | Colitis severity and <i>in vitro</i> release | [101] |
| | N/A | No | Eudragit S (M) | Aceclofenac | Paw edema severity | N/A | [139] |
| | N/A | No | Eudragit P-4135 F (M) | Calcitonin; carboxy-fluorescein | Plasma samples | Plasma concentrations and colon arrival time from literature | [79] |
| | N/A | No | P(LE-IA-MEG) (M) | Dexamethasone | Plasma samples | Plasma samples and <i>in vitro</i> release | [81] |
| | N/A | No | Eudragit S (C); Eudragit L (C); Eudragit RS100 (C) | Insulin | Plasma samples | N/A | [140] |
| | N/A | No | Eudragit L100-55 (M); Eudragit L (M); Eudragit S (M) | Celecoxib | Plasma samples; colitis severity | N/A | [141] |

TABLE 2 (Continued)

| Animal | | | Drug delivery system | | In vivo test | | Refs |
|---------------|-----------------------------------|------------------------------------|---|---|---|---|-------|
| Species | Rationale | pH values GI tract mentioned | pH-dependent matrix (M) and/or coating (C) | Drug/marker | Read out | Colon arrival determined with | |
| Rat and mouse | N/A | No | Eudragit S (C) | Budesonide | Tissue sections harvested | Tissue concentrations | [41] |
| | N/A | No | Eudragit S (C); alginate (M) | Ginger extract | Tissue sections harvested; clinical effects on colon cancer | Visual observation of ileocecal junction | [50] |
| | N/A | No | Eudragit S/Compritol (M) | 10-hydroxy-camptothecin; | Tissue sections harvested; microscopy; plasma samples | Plasma and tissue concentrations | [46] |
| | Disease model available | No | Eudragit S (M) | coumarin-6 Carboxy-fluorescein | Tissue sections harvested; plasma samples | Plasma and colon concentrations | [44] |
| | Disease model available | Yes | Eudragit P-4135F (M) | Ellagic acid with and without fluorescein | Tissue sections harvested; plasma samples | Plasma concentrations and visual observation of lower part of small intestine | [51] |
| | Based on practical considerations | No | Eudragit S (C) | IgY | Tissue sections harvested; plasma samples | Tissue concentrations | [142] |
| | N/A | No | Eudragit S (M); Eudragit L (M); Eudragit S/Eudragit L (M) | Insulin | Tissue sections harvested; plasma samples | Insulin content in microspheres from harvested tissue sections | [49] |
| | Disease model available | No | Eudragit S/PLGA ^b (M); Eudragit S (M) | Budesonide; coumarin-6 | Tissue sections harvested; plasma samples; microscopy; colitis severity | Plasma and tissue concentrations | [47] |
| | N/A | Yes | Eudragit S (M); Eudragit L (M); Eudragit L100-55 (M) | Prednisolone | Plasma samples | Not determined because of failure of system (pH threshold not reached in rat) | [143] |
| | N/A | No | Eudragit S/L and Surelease [®] (C) | Capecitabine | Tissue sections harvested | Tissue concentrations | [144] |
| | N/A | No | Eudragit S (C) | Curcumin and cyclosporine | Colitis severity | Colitis severity and <i>in vitro</i> release | [103] |
| | N/A | No | Eudragit S (M) | Tacrolimus | Colitis severity | <i>In vitro</i> release | [145] |
| | N/A | No | Eudragit S (C) | Budesonide; DiR ^c ; coumarin-6 | Tissue sections harvested; plasma samples | Visual observation of harvested tissue sections combined with plasma and colon concentrations | [45] |
| Rat and mouse | N/A | No | Pluronic/Polyacrylic acid (M) | Epirubicin; Toluidine Blue O | Tissue sections harvested; plasma samples; tumor size | Visual observation of harvested tissue sections | [54] |
| | N/A | No | P(CFC-MAA-MEG) (M) | Dexamethasone | Tissue sections harvested; plasma samples | Plasma and colon concentrations | [43] |

TABLE 2 (Continued)

| Animal | | | Drug delivery system | | In vivo test | | Refs |
|---------|--|------------------------------|--|---|---|--|-------|
| Species | Rationale | pH values GI tract mentioned | pH-dependent matrix (M) and/or coating (C) | Drug/marker | Read out | Colon arrival determined with | |
| Mouse | N/A | No | Eudragit S/Eudragit L (C) | Sulfasalazine | Paw edema severity | N/A | [146] |
| | N/A | No | P(CE-MAA-MEG) (M) | Mesalazine | Colitis severity | Colitis severity | [102] |
| | Disease model available | No | Eudragit S/PLGA (M) | Curcumin | Colitis severity | N/A | [147] |
| | N/A | No | Eudragit P-4135 F (M) | Tacrolimus | Colitis severity | N/A | [148] |
| | Disease model available | No | Eudragit S/PLGA ^b (M) | Curcumin | Tissue sections harvested, colitis severity | Visual accumulation in colon | [58] |
| | N/A | Yes | Eudragit FS 30 D (C); Eudragit L100-55 (C) | FITC-BSA; luciferase DNA plasmid; CpG vaccine | Tissue sections harvested | Cellular uptake in harvested tissue | [57] |
| | Disease model available | No | Eudragit FS 30 D/PLGA ^b (M) | Cyclosporine; DiR ^c | Tissue sections harvested; colitis severity | Visual observation of harvested tissue sections | [55] |
| | Disease model available | No | Eudragit S (C) | Budesonide; DiR ^c ; coumarin-6 | NIR spectroscopy; tissue sections harvested; disease severity | NIR spectroscopy and colon concentrations | [56] |
| | Disease model available | No | Eudragit S (M) | Curcumin | Colitis severity; plasma samples; fecal matter | Fecal concentrations | [48] |
| | Disease model available (mouse) and N/A (rabbit) | No | Eudragit P-4135F (M) | Enoxaparin | Plasma samples | Not possible because of negligible systemic absorption of enoxaparin | [149] |
| Rabbit | N/A | No | Eudragit S/Ethyl cellulose (C) | Metronidazole | Plasma samples | Plasma samples and colon arrival time from literature | [80] |
| | GI tract comparable to human | Yes | Eudragit S/Ethyl cellulose (C) | Theophylline | Radiography, plasma samples | Radiography and colon arrival time from literature | [62] |

^a Not available.^b Poly(lactic-co-glycolic acid).^c 1,1'-dioctadecyl-3,3',3'-tetramethylindotricarbocyanine iodide.

TABLE 3

Examples of colonic disease models in rabbits

| Disease | Disease induction | Refs |
|----------------------------|---|-----------|
| Colon cancer | VX2 carcinoma transplantation | [150,151] |
| | Azoxymethane subcutaneously | [152,153] |
| Irritable bowel syndrome | Moist heat, stress, and low-dose laxatives | [154] |
| | Colorectal distension with induced visceral hypersensitivity | [155,156] |
| Inflammatory bowel disease | Diluted acetic acid intrarectally | [157] |
| | Hapten intrarectally (e.g., 2,4,6-trinitrobenzenesulfonic acid or dinitrochlorobenzene) | [158–161] |
| | Inoculation with <i>Eimeria magna</i> oocytes intragastrically | [162] |
| | Degraded carrageenan orally | [163] |
| | Lipopolysaccharide intrarectally after 1% formalin enema | [164] |
| | Inoculation with Crohn's tissue homogenates intraileally | [165] |
| | Immune complex intravenously in combination with dilute formalin intrarectally | [166] |
| | Inoculation with <i>Bacteroides vulgatus</i> intra-appendiceal | [167] |
| | Dextran sodium sulfate orally | [168] |

identified (iib, iiib, and iv) (Table 4). One option is to retrieve the drug delivery system itself (iv) and to measure the drug content in the system, in which incomplete recovery indicates drug release. In the other approaches (iib and iiib), it is important that the drug is not extracted from the drug delivery system and that the luminal content is included in the assay either with or without the tissue. Drug release is then indicated by the presence of drug in the extract.

All methods described above measure drug content. Next to this approach, it is possible to detect the drug delivery system (e.g., microspheres or tablets) in harvested tissue samples visually or with a light microscope. In case of dissolving or eroding systems, these methods allow for the conformation of drug release from the disappearance of the drug delivery system [50–54]. When the drug delivery system is still present, drug release cannot be ruled out. More information is obtained when release can be visualized by fluorescence microscopy or NIR fluorescence microscopy, when a marker is included in the delivery system. For fluorescence microscopy, fluorescein or coumarin-6 have been used as markers and for NIR fluorescence imaging, 1,1'-dioctadecyl-3,3',3'-tetramethylindotricarbocyanine iodide (DiR), has been used [45,51,55,56]. Furthermore, cellular uptake of the marker/drug can be used to verify release [45,46,57,58].

TABLE 4

Overview of methods used to analyze harvested tissue samples

| Method | Drug extracted | | | Drug release indicated by | Applicable for drugs that are not absorbed |
|--------|----------------|-----------------|-----------------|----------------------------------|--|
| | Tissue | Luminal content | Delivery system | | |
| i | X | | | Presence of drug | |
| ii | X | X | X | Incomplete recovery ^a | |
| iib | X | X | | Presence of drug | X |
| iiia | | X | X | Incomplete recovery | |
| iiib | | X | | Presence of drug | X |
| iv | | | X | Incomplete recovery | X |

^a The total drug content is not retrieved.

When tissue sections are harvested, multiple animals are required. The consequence of this is that individual animals with variable GI transit times are compared, which makes interpretation of the data less reliable when the data are pooled. Moreover, the exact location of drug release cannot easily be determined, especially for multiparticulate formulations, such as microspheres, because they spread throughout the GI tract. Additionally, many animals have to be sacrificed to obtain data at sufficient time points. Furthermore, possible degradation or metabolism and/or transfer of the drug to the plasma have to be taken into account. The advantage of harvesting tissue samples is that it is possible to assess whether the drug has reached the colon. If the drug does not reach the colon, no drug would be measured or visually detected in the colonic tissue and/or luminal content. However, if no drug is measured or visually detected in the colonic tissue, it cannot be excluded with certainty that the drug has not reached the colon. More reliable conclusions can be drawn when plasma samples are taken at the same time points as the tissue samples, on the condition that the drug is immediately absorbed into the systemic circulation after release. The presence of drug in plasma indicates drug release and the presence in tissue sections might indicate the site of release.

Non-invasive imaging techniques

An attractive alternative to harvesting tissue samples, also in the light of the 3Rs (reduction, refinement, and replacement) for animal experiments [59], is the use of non-invasive imaging techniques. Most frequently used are radiography and fluoroscopy, followed by γ -scintigraphy and NIR fluorescence imaging [60–71].

Ionizing radiation, X-rays, are used in radiography and fluoroscopy to capture the images while in γ -scintigraphy the marker in the dosage form emits ionizing γ -radiation [72–74]. To visualize the dosage form in radiography or fluoroscopy, a contrast agent (e.g., barium sulfate) has to be integrated in the drug delivery system [73,74]. For radiography, fluoroscopy, and γ -scintigraphy, the cumulative ionizing radiation exposure has to be considered in the study design to ensure humane treatment of the animals. This is especially the case when animals are not sacrificed after the study, because radiation can cause long-term effects [75]. With NIR fluorescence imaging, no ionizing radiation is used and instead a fluorescent agent (e.g., DiR) is used as a marker compound [60,74]. The downside of NIR fluorescence imaging is that it suffers from a low resolution because of attenuation, scattering, and dispersion of the emitted light when it passes through tissues [60,74]. With all imaging techniques, the animals have to be restrained or brought under anesthesia to prevent blurred images, which causes

discomfort to the animal [75]. Radiography, fluoroscopy, and γ -scintigraphy are also used in clinical studies. The advantages and disadvantages of these methods were described in more detail in part I of this series [1].

Based on the evaluation of published animal studies in which non-invasive imaging techniques are applied, we suggest aspects that could be improved. Generally, only images were taken from one angle, generating a 2D image of the abdomen [60–71,73]. Reasons for taking only one image angle could be, for instance, reduction of radiation exposure, animal welfare, or practical considerations. Images from only one angle might lead to misinterpretation of disintegration and/or erosion of the drug delivery system or of the exact location of the drug delivery system in the GI tract because the depth cannot be determined. When the location of the different segments of the GI tract is determined with, for instance, a barium sulfate meal study in radiography or fluoroscopy studies, interpretation of the images is more straightforward [61,62,66,67,73,76]. Interpretation of images is more problematic in animal studies than in clinical trials because, in humans, the location of the large intestine is fixed in the body and the outline of the large intestine becomes visible when the marker is released [1]. In addition, the projection of the 2D images (e.g., dorsoventral) is often not given and the printed image quality and size is sometimes not optimal [60–62,64–70], which complicates interpretation of these images by the readers. Furthermore, image exposure and animal positioning could be improved, which would simplify interpretation of the images [60–64,66–69,71]. When these factors are not optimal and when only images from one angle are used, one should be reluctant with statements about colon targeting, because structures in the abdomen overlap and the exact 3D position cannot be determined with certainty.

Imaging techniques, when performed correctly, provide useful information about the position of a drug delivery system in the GI tract. However, these techniques do not provide information about the drug release from a system and, therefore, do not automatically give information about the ileocolonic targeting ability. This shortcoming can be overcome by combining imaging techniques with measuring plasma concentrations, especially when the drug and an imaging marker are combined in the same drug delivery system [60,62,67,71]. However, this is only valid for drugs that are absorbed over the entire GI tract.

Plasma samples

Drug and/or drug metabolite concentrations in plasma samples have been frequently used as a proof for ileocolonic drug delivery in animal studies (Table 2) [52,77–81]. However, plasma concentrations generally do not provide enough information to confirm ileocolonic drug delivery. An option is to compare the pharmacokinetic data obtained from the plasma curve to the colon arrival time, to determine whether the observed lag time of the system is sufficiently long to warrant targeting to the colon [62,77–80]. Kennedy *et al.* developed a method to measure the colonic arrival time, also called mouth-to-cecal transit time, in humans, using sulfasalazine [82], which has been validated by others [83]. This method is based on the fact that sulfasalazine is poorly absorbed by the GI tract but is converted by bacteria in the colon into sulfapyridine, which is subsequently absorbed [84,85]. Several researchers have used this method to circumvent interindividual variation

in colon arrival times [52,86]. It is also possible to circumvent the intraindividual variation in colon arrival time by combining sulfasalazine with a release marker (e.g., theophylline) in the drug delivery system [1,87]. In this method, theophylline is used as a marker for drug release, since it is absorbed over the entire GI tract. If there is no difference in plasma arrival time between theophylline and sulfapyridine, it indicates that the formulation released its contents into the colon. A downside of this method is that it only gives an answer to the question whether the drug delivery system releases its contents into the colon, but does not provide information as to the exact location in the colon. To determine the exact location of drug release, imaging techniques can be used in combination with the theophylline-sulfasalazine method. Furthermore, the pharmacokinetics and/or pharmacodynamics of the investigated drug might be influenced by either sulfasalazine or the release marker (in case the drug itself cannot be used as a release marker). In addition, sulfasalazine is degraded into not only sulfapyridine, but also mesalazine, which is a pharmacologically active compound used in the treatment of inflammatory bowel disease [82,88]. This should be taken into account if the compounds are combined into one drug delivery system.

Breath and urine samples

In addition to using plasma samples to determine the orocecal transit time, it is possible to use breath samples [89,90]. This method utilizes ^{13}C -urea (a stable isotope), which is metabolized by bacteria into $^{13}\text{CO}_2$ that is subsequently exhaled. In human volunteers, this method in combination with measuring ^{13}C -urea and ^{15}N -urea (an internal standard) in urine has been used to verify colonic drug delivery [1,91–93]. This principle could also be used in laboratory animals. Collection of urine in animals is possible by using a metabolic cage or by catheterization [94]. However, the use of metabolic cages generates a stressful environment for animals because of individual housing and the wire mesh floors, which can influence the therapeutic effect of a drug or exacerbate disease symptoms [94,95]. The latter stressor can be prevented by using hydrophobic sand for urine collection [96], but individual housing would remain an issue. In addition, catheterization is problematic because, among other issues, the catheter can be removed by the animal, and inserting the catheter is a stressor on its own [97]. The collection of breath samples is also problematic because of stress caused by handling or individual housing in a breath-test system [94,98]. The non-invasive character of the method makes it an ideal method to verify colonic drug delivery in humans [1]; however, because of the implications of urine and breath collection in laboratory animals, we do not recommend this method for animal studies.

Therapeutic effect

The last method, abundantly used to evaluate ileocolonic drug delivery systems in laboratory animals, is to determine a therapeutic effect (Table 2) [99–103]. However, the therapeutic effect as such does not directly answer the question whether the drug is in fact targeted to, and released into, the ileocolonic region. Therapeutic proteins or peptides given orally in an ileocolonic drug delivery system are an exception because they will be degraded in the upper GI tract. Therefore, they can only elicit a therapeutic effect if they are released in the colon [104]. In other cases, a

therapeutic effect should not be used as such to verify ileocolonic drug targeting, but should be used in combination with imaging techniques and plasma sampling to verify successful targeting. When the theophylline-sulfasalazine method is used or urine is collected with a metabolic cage, the influence on therapeutic effects of the drug under investigation should first be taken into account before conducting the experiments.

Overall, the determination of the therapeutic effect is valuable, because it can answer the questions whether the drug delivery system can improve clinical symptoms and whether it is superior to nontargeted drug delivery systems.

Concluding remarks

For testing novel drugs for ileocolonic delivery and/or for pH-dependent ileocolonic drug delivery systems containing novel excipients, animals have to be used. To obtain ileocolonic drug delivery, a sharp distinct pH peak in the terminal ileum is crucial, thus a similar pH to humans is not essential. However, if the pH profile of the GI tract in animals is similar to that of humans, established ileocolonic drug delivery systems can be used to test novel drugs. When novel pH-dependent excipients have to be tested, a pH profile similar to that in humans is a prerequisite to obtain ileocolonic drug delivery in humans. In this respect, the rabbit is the most appropriate animal species compared with other frequently used laboratory animals, because their GI pH values are most similar to those of the human GI tract. However, not only the pH values of the GI tract, but also the desired disease model and the size of the delivery system have to be taken into account. If the rabbit cannot be used, then the pig, rat, and dog might be suitable alternatives, on the condition that the pH values of individual

animals are verified first. To properly draw conclusions from the obtained efficacy data, ileocolonic drug delivery must be verified. The different methods used for this verification all have specific advantages and limitations, thus the optimal method should be determined for each study. Non-invasive imaging techniques in combination with plasma sampling can be used if the therapeutic effect of a novel drug is investigated. When a novel excipient in the drug delivery system itself is subject of investigation, the theophylline-sulfasalazine method is an elegant way to verify colonic drug delivery.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: HWF is one of the inventors of a patent (WO 2007/013794) describing a method for colon targeting, which is held by his employer. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The literature search was partially funded by Janssen Pharmaceutica. Janssen Pharmaceutica had neither role in study design, in the collection, analysis, and interpretation of data, nor in the writing of the report and in the decision to submit the paper for publication.

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